# Detection the Genetic Polymorphisms of the Programmed Cell Death Gene *PD1.3 G/A (rs11568821)* in Coronavirus Disease Patients

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## Abstract

**Background:** Programmed cell death-1 protein (PD-1, Pcd1), a receptor from the CD28/CTLA-4 group, inhibits antigen receptor signaling by attracting protein tyrosine phosphatase in response to an interaction with any of the two ligands, PD-L1 or PD-L2. Human phenotypic variations can be attributed to both genetic and environmental influences. **Objectives:** Because SNPs (single nucleotide polymorphisms) are not absolute indicators of disease progression, in this work, the PD-1 gene polymorphism in coronavirus disease (COVID-19) patients was examined to assess the presence of SNPs in viral infections, in particular COVID-19 is the main goal of this investigation. **Materials and Methods:** Sixty confirmed COVID-19 patients were recruited to this study; 30 of them had severe COVID-19, whereas the other patients showed moderate sickness, who were admitted to the COVID-19 specialized ward in Salahuddin province, and all of them were over the age of 18 years. Also 30 healthy subjects were recruited for the purpose of the comparison. Blood was drawn from all the subjects for polymerase chain reaction (PCR) using the restriction fragment length (RFLP)-PCR for the assessment of the G/A SNP genotype of PD1.3 and ELISA test for the estimation of PD-1 and PD-L-1 serum level. **Results:** The examined *PD-1* SNPs did not correlate with the incidence of COVID-19, according to a data analysis. Both the dominant and recessive models used in the research failed to detect a connection with the risk of COVID-19 severity. The PD1.3 genotypes frequency between the two groups did not show significant differences ($P > 0.05$). Only AG was substantially and mainly linked to COVID-19 susceptibility. This study compared the concentrations of immune check point inhibitors PD-1, PD-L-1 to find possible association with genotype frequency, and approved that PD-1 did not have any significant differences in the three groups of genotypes, whereas the difference was highly significant (0.048) in PDL-1 and AA genotype. **Conclusion:** Among all calculated haplotypes were unrelated to the disease's prognosis ($P > 0.05$) concluded that the frequency of AA genotype in patients group decreed the expiation of PD-L-1, leads to immune inhibitions. Future research may clarify the relationship between some immune checkpoint molecule polymorphisms.

**Keywords:** COVID-19, PD-1, polymorphisms, RFLP-PCR, SNP

## Introduction

A respiratory condition known as coronavirus disease 2019 (COVID-19) is caused by the SARS-CoV-2 coronavirus, which initially appeared in the late-2019.[1] The virus has a 29.8 kb single-stranded RNA genome and is a member of the Beta coronavirus genus, which is related to the bat coronaviruses.[2] The virulent factor is mostly facilitated by its human spike receptors. Individuals are more susceptible to severe manifestations of COVID-19 due to a number of reasons, most notably the cellular immune response.[3] The disparity of lymphocytes and mediator molecules leads to infection-related dysregulated reactions.[4]. On chromosome 2, the PD-1 gene is polymorphic, and single-nucleotide polymorphisms (SNPs) in this gene have been linked to autoimmune disorders and cancer susceptibility.[5]

The clinical severity of COVID-19 and the PD-1/PD-L1 has received attention because the data shown that PD-L1

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serum levels may be a prognostic factor.[6] Additionally, there is a proof of an increase in the expression of CD4 positive and CD8 positive T cells' of PD-1 patients with COVID-19, and PD-1 blocking medications, bolster the body's defenses against the virus.[7] There is growing evidence that in critically sick patients, hyperinflammation is linked to C-reactive protein (CRP) and lymphocytes.[8] These findings suggest that the pathophysiology of COVID-19 may be significantly influenced by a cytokine storm.[8] The PD-1/PD-L1 signaling pathway is responsible for balancing the stimulatory and inhibitory signals necessary for successful immune responses against microbes as well as has been linked to a variety of diseases and self-tolerance.[9] The PD-1 ligand is expressed on macrophages and dendritic cells (DCs) and has a regulatory function when these cells are activated. Some immune cells, such as T cells, NK cells, B cells, and monocytes, express PD-1, whereas DCs, macrophages, vascular endothelial cells, and other cells express PD-L-1, which is the ligand for PD-1.[10] This study aimed to find possible association of these immune check point inhibitors PD-1/PD-L-1 with allele frequency in COVID-19 patients. And, for knowing the correlation between the genetic variation in a specific gene and autoimmune indicators and their relationship to the severity of infection, we can conclude that these indicators can increase or decrease the severity of the infection in patients of Corona and the extent of their impact on public health.

## Materials and Methods

### Subjects and blood samples collection

This study was carried out in the form of a cross-section from December 2021 to March 2022. A total of 90 whole blood samples (5 mL) were collected from 60 confirmed COVID-19 patients (54 male and 6 female patients) and 30 healthy controls (15 male and 15 female patients). Any patients who have cancer, individuals who are receiving treatment for autoimmune diseases, and also women who are pregnant or nursing were excluded. All diagnosed COVID-19 patients, who were confirmed positively by SARS-CoV-2 nucleic acid (RT-PCR) by using specimens derived from their oropharyngeal swabs, were included. Thirty patients had severe COVID-19, whereas the remaining 30 had moderate illness that was clinically determined to be present by a positive nasopharyngeal swab and validated by PCR. According to WHO criteria of severity, any adolescent or adult person with one or more of the following symptoms in addition to clinically obvious pneumonia (fever, cough, dyspnea) is regarded to have a severe disease with the following conditions such as breathing more than 30 times per minute, acute respiratory discomfort, and 90% SpO2 in room air. Even though the diagnosis is typically made on the basis of clinical considerations, a moderately infected adult who exhibits the typical symptoms of pneumonia (fever, cough, dyspnea, and rapid breathing) but does not exhibit symptoms of a severe infection, such as SpO2 levels that are lower than 90% on room air, SpO2 in the air of the room is 90%, And illness without signs of pneumonia or hypoxia shall be assumed to have the COVID-19 symptoms.[11] The patients were admitted to the COVID-19 specialized ward in Salahuddin Hospital, and all of them were over the age of 18 years. The individuals' peripheral venous blood was drawn and put into tubes that were coated with EDTA.

### DNA extraction

Utilizing the Relax Gene Blood DNA System and following the manufacturer's instructions (TIANGENBIOTECH, Beijing, China), genomic DNA was extracted for the enrolled individuals.

By using spectrophotometer to measure the absorbance at 260 nm in order to get an accurate reading of the DNA concentration (one unit of absorbance at 260 nm corresponds to 50 g of genomic DNA per mL), DNA was dissolved in buffer CE and kept at a temperature of –20°C for long-term preservation. By using spectrophotometry to determine absorbance at 260 nm, DNA quality and amounts were confirmed.

### RFLP-PCR

All groups were exanimated for *PD1.3 A/G (rs11568821)* gene polymorphism by using restriction fragment length polymorphism-PCR (RFLP-PCR Premix Bioneer, Korea).

PCR-RFLP was used to investigate the *PD-1.3 A/G* polymorphism. The PCR primers were designed using a program named Primer Premier 5.0. according to manufacturer's instructions. The primer sequences for *PD-1* were 5′-CCCCAGGCAGCAACCTCAAT-3′ (forward) and 5′-GACCGCAGGCAGGCACAT-3′ (reverse).

The digestion product was no cutting of Allele G:180, the cutting allele was A:123 bp and 57 bp, and the final product of heterozygous GA genotype will be 180 bp 123 bp and 57 bp.

After completing the method for isolating the sample, 3 µL of it was taken and mixed it with 2 µL of purple gel loading dye, and then this mixture used to fill the well of an electrophoresis gel made of 1% agarose gel. The process was carried out at 5 volts per cm for 30 min. The following circumstances[12] were used for the DNA amplification denaturation for 5 min at 95ºC, annealing for 6 min at 56ºC, an extension for 30 s at 72ºC, and a final extension for 7 min at 72ºC. The *PD-1* gene polymorphism was looked for using a 25 µL final extract volume. Following gel electrophoresis, digestion with the restriction enzyme PST I was done to cut the band at the site A:123 bp and 57 bp and ethidium bromide monitoring, the amplified PCR products were tested.

### ELISA assay

An ELISA test (Mindray, China) to detect concentration of immune check point inhibitors (PD-1 and PD-L-1) was used. The test included a sandwich immunodetection technique, in which antigen-antibody complexes developed.

## Statistical analysis

For the analysis of the cases and the controls, Hardy-Weinberg equilibrium (HWE) tests were conducted. Direct gene counting was used to assess the genotype and allele frequencies in the afflicted and control individuals. The SPSS version 18 program was used to conduct the statistical analysis. The Social Sciences Statistical measurements, the chi square test, and Fisher's exact test were used to examine the frequencies of alleles and genotypes in cases and controls. Each reported *P* value had a two-tailed distribution, was statistically significant, and had a probability of 0.05.

## Ethical approval

The study was conducted was carried out with patients' verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 13547 on December 18, 2021.

# Results

The individuals enrolled in this study were divided by gender into 54 (90%) male patients and 6 (10%) female patients in patients' group; and 15 (50%) male patients, with 15 (50%) female patients in the control group.

The genotype frequency distribution in the control groups was in accordance with HWE. The frequencies of the AA, GG, and GA genotypes were not statistically different between the patients and the healthy individuals ($P$ = 0.817), although AA seemed to be more in the patients' groups.

Additionally, the control group had a high frequency of GG (43.3%) and GA (36.7%) genotypes as compared to AA (20%) genotypes. Whereas the distribution in moderate COVID-19 patients was GA (30 %), GG (36.7 %), and AA (23.3%), but GA (36.7 %) and GG (40 %) were noted in severe COVID-19 patients as shown in Table 1 and Figures 1 and 2.

In COVID-19 patients, the means ± SD distribution of selected parameters according to genotyping (AA, GA, and GG) showed that *PD-1* did not show any significant differences in the three genotypes, whereas the difference was highly significant in PD-L-1. The mean of PD-L-1 in AA genotype had the lowest P value (0.048), which is shown in Tables 2 and 3.

The level of the same parameters was observed in the three genotyping in control subjects to understand the effect of SNP on selected parameters, and there were no observable differences between the means of PD-1 and PDL-1 in AA, GA, or GG genotypes [Table 4].

**Table 1: Genotypes of *PD-1.3* A/G (rs1156882) single nucleotide polymorphism (SNP) in Patients with SARS-COV-2 and healthy controls**

| | | Severe COVID-19 | | Moderate COVID-19 | | Control | | *P* value |
|---|---|---|---|---|---|---|---|---|
| | | No | % | No | % | No | % | |
| Genotype | AA | 7 | 23.3 | 10 | 33.3 | 6 | 20.0 | 0.817 |
| | GA | 11 | 36.7 | 9 | 30.0 | 11 | 36.7 | |
| | GG | 12 | 40.0 | 11 | 36.7 | 13 | 43.3 | |

* Using the Pearson Chi-square test ($\chi^2$ test) at the 0.05 level, there were significant differences among the percentages

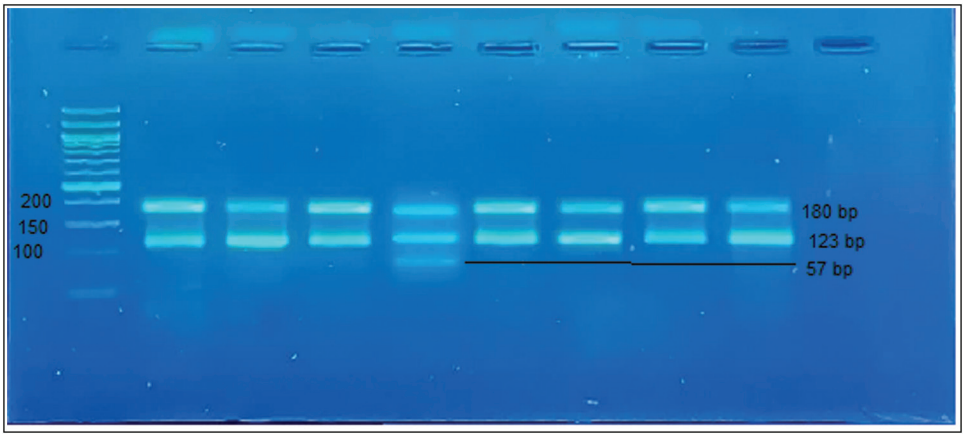


**Figure 1:** Agarose gel electrophoresis the *PD-1* gene, of COVID-19 patients, amplified by PCR and subjected to (RFLP) followed by separation on 2% agarose gel stained with ethidium bromide, and visualized under UV light, at 5 volts per cm for 30 min

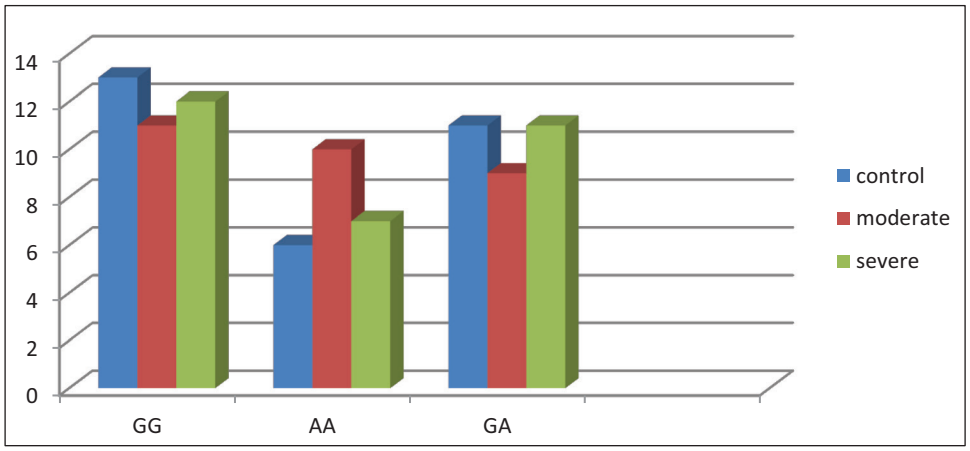


**Figure 2:** Guanine Guanine GG, Adenine Adenine AA, Guanine Adenine GA Genotypes of patients with SARS-COV-2 compared to the control group

**Table 2: The relationship between genotypes and ELISA test for PD-1 and PD-L-1 in COVID-19 patients**

| COVID-19 | Genotype | | | *P* value |
|---|---|---|---|---|
| | AA | GA | GG | |
| PD-1 (ng/mL) | 1.64 ± 1.43 | 2.81 ± 2.44 | 1.95 ± 1.32 | 0.124 |
| PDL-1 (ng/mL) | 3.78 ± 1.58 | 7.16 ± 4.17 | 6.57 ± 5.56 | 0.048^ |

^ ANOVA-test significant difference between more than two independent means at the 0.05 level

**Table 3: Dilution of stander solution for PD-1, PD-L-1 of ELISA kit provides one standard original concentration**

| | | |
|---|---|---|
| 60 ng/mL | Standard No. 5 | 150 µL original standard + 150 µL standard diluents |
| 30 ng/mL | Standard No. 4 | 150 µL original standard No. 5 + 150 µL standard diluents |
| 15 ng/mL | Standard No. 3 | 150 µL original standard No. 4 + 150 µL standard diluents |
| 7.5 ng/mL | Standard No. 2 | 150 µL original standard No. 3 + 150 µL standard diluents |
| 3.75 ng/mL | Standard No. 1 | 150 µL original standard No. 2 + 150 µL standard diluents |

**Table 4: The relation between genotypes and PD-1 and PDL-1 in in healthy controls**

| Control | Genotype | | | *P* value |
|---|---|---|---|---|
| | AA | GA | GG | |
| PD-1 (ng/mL) | 0.77 ± 0.32 | 0.69 ± 0.24 | 0.55 ± 0.29 | 0.232 |
| PDL-1 (ng/mL) | 0.72 ± 0.56 | 0.89 ± 0.27 | 0.85 ± 0.30 | 0.639 |

^ ANOVA-test significant difference between more than two independent means at the 0.05 level

## Discussion

At the level of single nucleotide polymorphisms (SNPs) of the genome, this study investigated the relationship between PD-1 genotypes and the risk of SARS-COV-2, and some immune checkpoint inhibitors included PD-1, PD-L-1 in a group of patients and controls, and subsequently pathogenic variables. This study found a significant link between SARS-COV-2 and PD-L-1 particularly AA, suggesting that poor PD-1-associated immunomodulation contributes to the development of SARS-COV-2. To our knowledge, this is a first-of-its-kind study that illustrates the significance of PD-1 in the upkeep of immunological tolerance in this context. Previous research has demonstrated a correlation between PD-1 gene polymorphisms and autoimmune disorders such as ankylosing spondylitis, rheumatoid arthritis, and systemic lupus erythematous.[13]

Another previous research showed that genetic variations play a central role in the susceptibility to various diseases, including cancer, autoimmune disorders, and infectious diseases like COVID-19. It appears that a variety of genetic variations and environmental factors influence the pathogenic outcome of COVID-19.[14] It is essential to know the elements that are implicated in the disease in order to understand the etiology and pathogenic processes connected with the SARS-CoV-2

sickness. Additionally, this might help identify COVID-19 disease risk, allowing for more precise prevention. The host response susceptibility to SARS-CoV-2 disease can cause a wide range of pathophysiological problems,[13,15] Additionally, it appears that immune cell genetic diversity may affect protection against SARS-CoV-2. This study looked for the correlation between the *PD-1* gene polymorphism and the ligand PD-L1 in COVID-19 severity.

Whereas individual mutations have been studied, they may not be linked to a person's propensity for certain diseases; nevertheless, a combination of mutations or SNPs may change a protein's structure or function, further reducing the response of immunity. The PD-1 gene allelic study showed no correlation between this gene and COVID-19 vulnerability. Accordingly, this study was conducted to look for a correlation between COVID-19 severity and the *PD-1* gene polymorphisms, and as far as we know, no other research has been done in this field. However, there is a proof that certain types of carcinomas and viral infections are related to *PD-1* gene polymorphisms.[16,17] The study, which looked at the effects of hepatitis C infection and PD-1gene variants, concluded that the genotypes or alleles of PD-1.3 G/A and PD1.5 C/T did not impact a patient's chance of contracting an infection.

## Conclusion

Despite various limitations and biases, the current study findings had no statistical correlation between COVID-19 infection with any of the genotypes' frequencies in the studied groups. The difference between PD-L-1 and the AA genotype was highly significant, according to the means ± SD distribution of selected parameters and according to genotyping (AA, GA, and GG) from which we concluded that the frequency of AA genotype in patients group decreed the expiation of PD-L-1 leads to immunomodulation. When trying to encourage genetic profiling for setting up individualized therapies to improve COVID-19 treatment options, an individual's overall grasp of their distinct polymorphisms may aid to more fully explain COVID-19 outcomes. Future research may clarify the relationship between some immune checkpoint molecule polymorphisms and describe its relationship with disease prognosis.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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